Virology **266,** 227–236 (2000)
doi:10.1006/viro.1999.0088, available online at http://www.idealibrary.com on IDEAL®

Identification of Two Major Virion Protein Genes of White Spot Syndrome Virus of Shrimp

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Received August 25, 1999; returned to author for revision October 28, 1999; accepted November 8, 1999

White Spot Syndrome Virus (WSSV) is an invertebrate virus, causing considerable mortality in shrimp. Two structural proteins of WSSV were identified. WSSV virions are enveloped nucleocapsids with a bacilliform morphology with an approximate size of 275 × 120 nm, and a tail-like extension at one end. The double-stranded viral DNA has an approximate size 290 kb. WSSV virions, isolated from infected shrimps, contained four major proteins: 28 kDa (VP28), 26 kDa (VP26), 24 kDa (VP24), and 19 kDa (VP19) in size, respectively. VP26 and VP24 were found associated with nucleocapsids; the others were associated with the envelope. N-terminal amino acid sequences of nucleocapsid protein VP26 and the envelope protein VP28 were obtained by protein sequencing and used to identify the respective genes (*vp26* and *vp28*) in the WSSV genome. To confirm that the open reading frames of WSSV *vp26* (612) and *vp28* (612) are coding for the putative major virion proteins, they were expressed in insect cells using baculovirus vectors and analyzed by Western analysis. A polyclonal antiserum against total WSSV virions confirmed the virion origin of VP26 and VP28. Both proteins contained a putative transmembrane domain at their N terminus and many putative *N*- and *O*-glycosylation sites. These major viral proteins showed no homology to baculovirus structural proteins, suggesting, together with the lack of DNA sequence homology to other viruses, that WSSV may be a representative of a new virus family, Whispoviridae.

Key Words: Penaeus monodon; White Spot Syndrome Virus; virion proteins; *vp26* and *vp28* genes; taxonomy.

INTRODUCTION

White Spot Syndrome Virus (WSSV) is a major viral disease agent in shrimp in large coastal areas of Southeast Asia and North America. The virus has a wide host range among crustaceans (Flegel, 1997) and distinctive clinical signs (white spots) in penaeid shrimps. There is little genetic variation among WSSV isolates from around the world (Lo *et al.,* 1999). Electron microscopy (EM) studies showed that the virions are enveloped and have a bacilliform shape of ~275 nm in length and 120 nm in width with a tail-like appendage at one end (Wongteerasupaya *et al.,* 1995). Nucleocapsids, which have lost their envelope, have a cross-hatched appearance and a size of ~300 × 70 nm (Wongteerasupaya *et al.,* 1995). This virion morphology, its nuclear localization, and its morphogenesis are reminiscent of baculoviruses in insects (Durand *et al.,* 1997). Originally, WSSV was classified as an unassigned member of the Baculoviridae (Francki *et al.,* 1991). At present WSSV is no longer accepted into this family (Murphy *et al.,* 1995) due to its unusually wide host range and lack of molecular information. The double-stranded viral DNA is 290 kb, as determined from restriction endonuclease analysis (Yang *et al.,* 1997), but sequence information is almost entirely lacking.

Recent analysis of the WSSV DNA revealed the presence of putative genes for the large and the small subunit of ribonucleotide reductase (RR1 and RR2) (van Hulten *et al.,* 2000). This enzyme is often found encoded by large DNA viruses, including baculoviruses (van Strien *et al.,* 1997). The genes for RR1 and RR2 are the first ORFs in the WSSV genome for which a putative function could be assigned. To study the relationship of WSSV to other large DNA viruses, eukaryotes, and prokaryotes, phylogenetic trees were constructed using the amino acid sequences of RR1 and RR2. This analysis showed that WSSV belongs to the eukaryotic branch of an unrooted parsimonious tree and further indicated that WSSV and baculovirus RRs do not share an immediate common ancestor (van Hulten *et al.,* 2000).

Other genes that are highly conserved in related viruses are those coding for viral capsid or envelope proteins (Murphy *et al.,* 1995). These genes are therefore often used to study virus relatedness. The major capsid proteins were found to be highly conserved among members of the families Iridoviridae, Phycodnaviridae, and African swine fever virus (Tidona *et al.,* 1998) and were found to be a suitable target for the study of viral evolution of these DNA viruses (Tidona *et al.,* 1998). This is also the case in Poxviridae (Sullivan *et al.,* 1994). In

Sequence data from WSSV 26 kDa (VP26) and the WSSV 28 kDa (VP28) protein genes have been deposited in GenBank under Accession Nos. AF173992 and AF173993.

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0042-6822/00 $35.00

Baculoviridae, 70% of the structural virion proteins are well conserved (Ahrens *et al.,* 1997; Gomi *et al.,* 1999), and many baculovirus structural proteins share antigenic determinants (Smith and Summers, 1981). In plant RNA viruses, the phylogeny for several different families can also be elucidated based on the viral capsid genes (Dolja and Koonin, 1991; Dolja *et al.,* 1991).

Considering the conserved nature of viral capsid proteins and their use in virus taxonomy and phylogeny, virion proteins of WSSV were analyzed and two genes were identified on the WSSV genome. Their viral identity was further investigated by overexpression of these proteins in the baculovirus-insect cell expression system (Summers and Smith, 1987) and by Western analysis using a polyclonal against purified WSSV virions. The two WSSV virion proteins showed no homology to known baculovirus proteins or to other proteins in databases. This result gives further support to the proposition that WSSV might be a representative of a new virus family (van Hulten *et al.,* 2000).

RESULTS

Identification of WSSV virion proteins

Penaeus monodon shrimp were infected with WSSV by intramuscular injection of a purified virus preparation. Four days after infection virus was isolated from the haemolymph of the infected animals. As a negative control, haemolymph was also taken from uninfected shrimps. These preparations were analyzed by electron microscopy for the presence and purity of WSSV virions. In the samples of uninfected animals, no virus particles were observed, but in samples of the infected animals, many mainly enveloped virions were observed (Fig. 1a).

When the viral envelope was removed from the virus particles after treatment with N-P40, the purified nucleocapsids (Fig. 1b) had a cross-hatched appearance characteristic for WSSV nucleocapsids (Durand *et al.,* 1997). The proteins of the enveloped virions, the nucleocapsids and the envelope fraction were separated by SDS–PAGE (Fig. 1c), and for the nucleocapsid and the envelope fraction, a Western blot using WSSV antiserum was prepared (Fig. 1d). Three major protein bands in the range of 67–78 kDa were present in the shrimp haemolymph and copurified with the virions. In the purified WSSV virions (Fig. 1c), four major peptides were identified with an apparent molecular mass of 28 kDa (VP28), 26 kDa (VP26), 24 kDa (VP24), and 19 kDa (VP19), respectively. Several less prominent bands were also observed, approximately six of which are located in the range of 30–65 kDa, and at least seven weak protein bands range from 86 to 130 kDa in size. No bands of the major WSSV proteins VP28 and VP19 were present in the lane containing WSSV nucleocapsids but were weakly visible in the lane containing the envelope fraction (Fig. 1c) and clearly visible in the Western blot (Fig. 1d). They therefore seemed to be derived from the viral envelope or tegument. VP26 and VP24 were present, both in the nucleocapsids and in the virions (Fig. 1c), suggesting that they are derived from the nucleocapsid. In the Western blot, VP26 is clearly visible; however, VP24 was only weakly visible as this protein does not react very well with the WSSV antiserum. A low background is visible from the envelope-derived proteins, as these react very strongly with the WSSV antiserum.

The protein from the SDS–PAGE gel was transferred to a polyvinylidene difluoride membrane by semi-dry blotting, and the major viral protein bands were excised and sequenced. More than 40 amino acids were sequenced from the N terminus of VP28 and VP26 (bold-faced in Figs. 2a and 2b, respectively). The VP26 N-terminal sequence was M E F G N L T N L D V A I I A I L S I A I I A L I V I M V I M I V F N T R V G R S V V A N. N-terminal sequencing of VP28 gave the amino acid sequence M D L S F T L S V V S A I L A I T A V I A V F I V I F R Y H N T V T K T I E t H s D, of which the threonine at position 39 and the serine at position 41 are uncertain. Both N-terminal sequences are hydrophobic (Fig. 3).

Localization and sequence of the 26-kDa protein gene

Partial WSSV genomic libraries of *Hin*dIII, and *Bam*HI were constructed in pBluescript-SK+ (van Hulten *et al.,* 2000), and terminal nucleotide sequences were obtained from many WSSV fragments. The nucleotide sequence encoding the N-terminal amino acid sequence of VP26 is present near a terminus of a 6-kb *Bam*HI fragment (Fig. 2c). The sequence surrounding the methionine start codon (AAA*ATG*G) conformed with the Kozak rule for efficient eukaryotic translation initiation (Kozak, 1989). Only 49 nucleotides of the untranslated leader of *vp26* could be determined, extending toward the terminal *Bam*HI site (Fig. 2c).

The 6-kb *Bam*HI fragment contained an open reading frame of 615 nt, including those encoding the N-terminal amino acids of VP26 (Fig. 2a). A polyadenylation consensus (polyA) signal is present 34 nt downstream of the translational stop codon of *vp26*. The *vp26* encodes a protein of 204 amino acids with a theoretical size of 22 kDa. The putative protein is basic with an isoelectric point of 9.3. Three potential sites for *N*-linked glycosylation (N-{P}-[ST]-{P}) are present, and three putative *O*-glycosylation sites were predicted using the program NetOglyc (Hansen *et al.,* 1998) (Fig. 2a). Fourteen possible phosphorylation sites ([ST]-X-X-[DE] or [ST-X-[RK]) were found, but no other motifs present in the PROSITE database.

Hydrophobicity analysis of the amino acid sequence of VP26 showed that a strong hydrophobic region was present at the N terminus of the protein (Fig. 3a). This

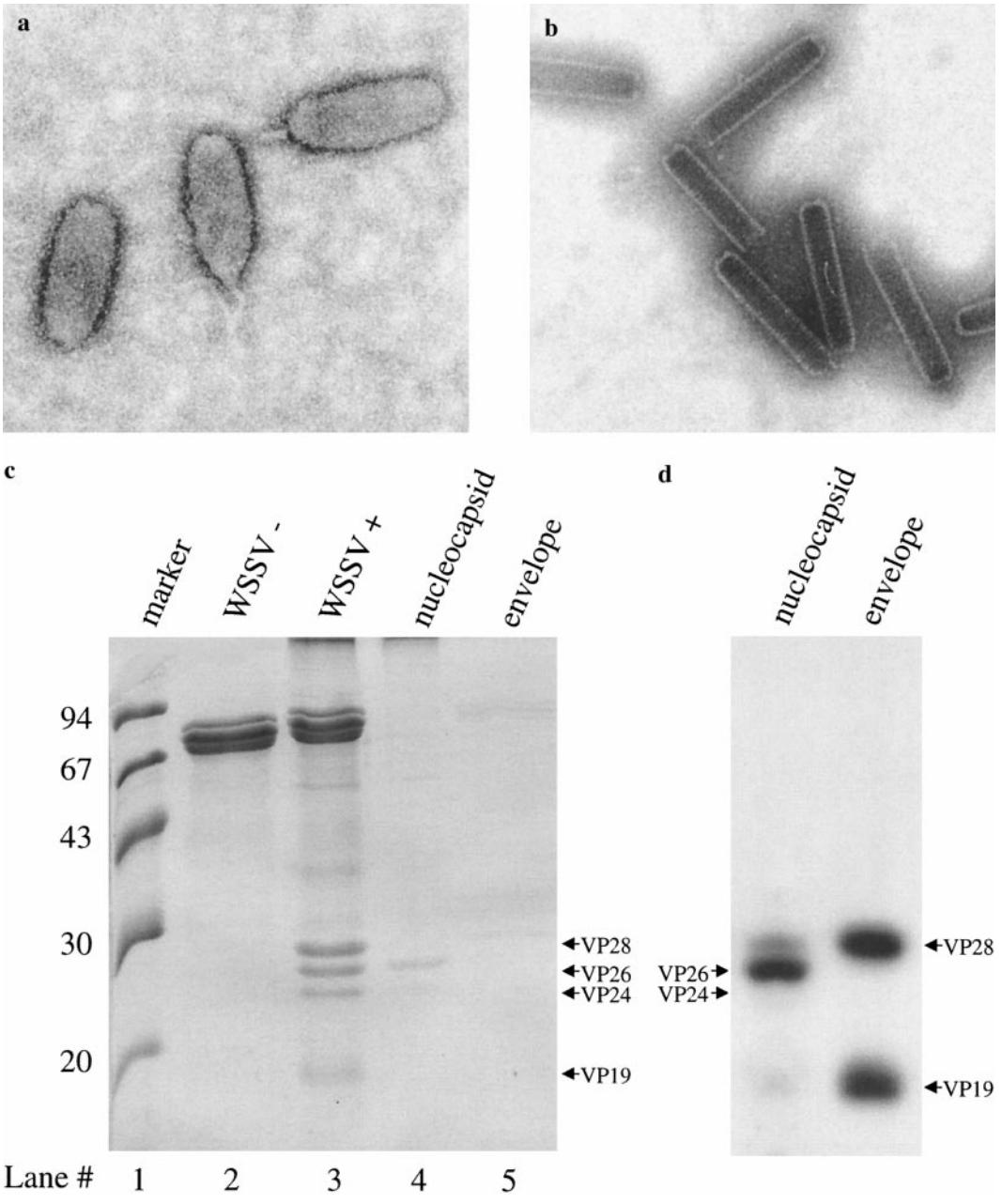

FIG. 1. (a) Electron microscopic view of negatively stained intact WSSV virions, (b) of negatively stained WSSV nucleocapsids. (c) Fifteen percent Coomassie-Brilliant-Blue-stained SDS–PAGE gel of purified WSSV. Lane 1: low molecular weight protein marker. Lane 2: mock purification from uninfected shrimps. Lane 3: purified WSSV particles. Lane 4: purified WSSV nucleocapsids. Lane 5: WSSV envelope fraction. (d) Western blot of the nucleocapsid and envelope fraction of C. WSSV polyclonal antiserum is used and detection is performed with the ECL kit.

region contained a putative transmembrane anchor consisting of an α-helix formed by amino acids 12–34. The anchor was followed by a positively charged region with two arginines, suggesting that the C-terminal part of the protein is on the cytoplasmic side (Sonnhammer *et al.,* 1998). Besides the transmembrane-spanning α-helix, a potential β sheet was found at position 127–141 using the algorithm of Garnier *et al.* (1978). Only one cysteine was present in the protein, indicating that no intraprotein disulfide cross-links can be formed. This cysteine was located in the C-terminal part of the protein.

Localization and sequence of the 28-kDa protein gene

The coding sequence of VP28 could not be determined from sequence analysis of the WSSV DNA fragment termini. Based on the N-terminal protein sequence of VP28 a set of degenerated primers was developed. The forward primer was 5′ CA*GAATTC*TCDATNGTYTTNGT-

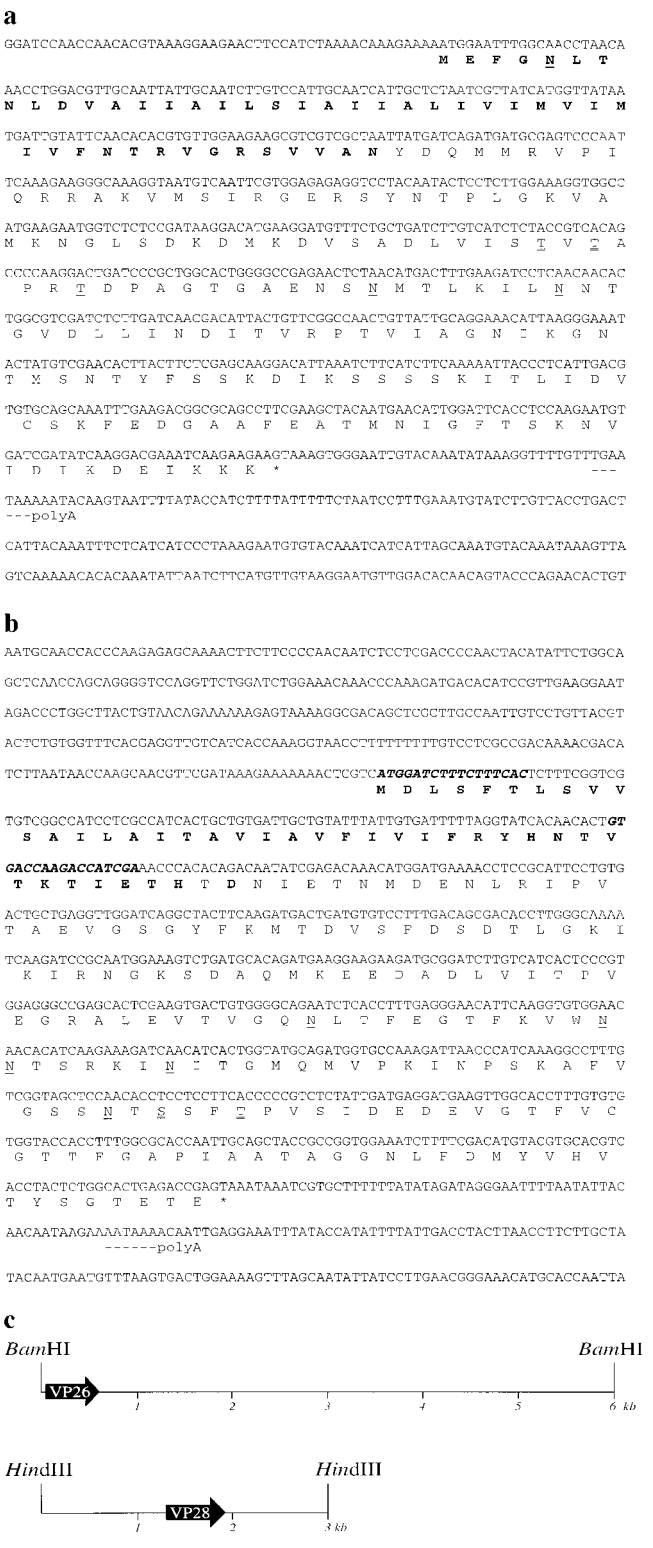

FIG. 2. Sequence of WSSV VP26 and VP28. Figure shows nucleotide and protein sequence of VP26 (a) and of VP28 (b). The N-terminal sequenced amino acids are bold-faced; the location of putative *N*-glycosylation sites is underlined and of *O*-glycosylation sites is double underlined. The nucleotide sequence of degenerated primer positions on VP28 is in bold and italics. (c) Location of VP26 and VP28 on WSSV genomic fragments.

NAC 3′ and the reverse primer was 5′ CA*GAATTC*ATG-GAYYTNWSNTTYAC 3′. *Eco*RI sites (italics) were incorporated into the primers. The location of the primers in the final sequence is indicated in Fig. 2b. PCR was performed using WSSV genomic DNA as template. A 128-bp-long fragment was obtained and, after purification from a 2.5% agarose gel, cloned into pBluescript SK+ and sequenced. The nucleotide sequence of this PCR product encoded the N-terminal protein sequence of WSSV VP28, and this 128-bp fragment was used in a colony lift assay (Sambrook *et al.,* 1989) on several WSSV plasmid libraries. A 3-kb *Hin*dIII fragment hybridized with this fragment and so was further analyzed.

The complete *vp28* ORF and a promoter region of this gene was found on this 3-kb *Hin*dIII fragment (Fig. 2b). The methionine start codon (GTC*ATG*G) was in a favorable context for efficient eukaryotic translation initiation (Kozak, 1989). In the promoter region, no consensus TATA box was found, but stretches of A/T rich regions were present. A polyA signal was observed 55 nt downstream of the translation stop codon. The *vp28* ORF coded for a putative protein of 204 amino acids, including the N-terminally sequenced amino acids of VP28. The theoretical size of this protein was 22 kDa, and it had an isoelectric point of 4.6. Five potential sites for *N*-linked glycosylation (N-{P}-[ST]-{P}), two sites for *O*-glycosylation (Hansen *et al.,* 1998) (Fig. 2b) and 9 possible phosphorylation sites ([ST]-X-X-[DE] or [ST-X-[RK]) were found within VP28. No other motifs present in the PROSITE database were found in VP28.

Computer analysis of the 204 amino acids showed that a strong hydrophobic region was present at the N terminus of VP28 (Fig. 3b), including a putative transmembrane α-helix formed by amino acid 9–27. As in VP26, this transmembrane anchor sequence is followed by a positively charged region, suggesting that the protein might have an outside to inside orientation. At the C-terminal part of the sequence, another hydrophobic region, which might constitute a transmembrane sequence, was found. However, the algorithm of Garnier *et al.* (1978) did not predict an α-helix at this position in VP28. The algorithm predicted another α-helix at position 89–99, but no β sheets along the protein. As in VP26 only one cysteine was present in VP28. This cysteine was also located in the C-terminal part of the protein.

Expression and analysis of recombinant *vp26* and *vp28*

Vp26 and *vp*28 were expressed in the baculovirus insect cell system to determine whether the *orf*s represent the major WSSV structural virion proteins and to check the immunoreactivity of these proteins with a WSSV- and *Ac*MNPV-specific polyclonal anitisera, respectively. The Bac-to-Bac system (GIBCO BRL) was

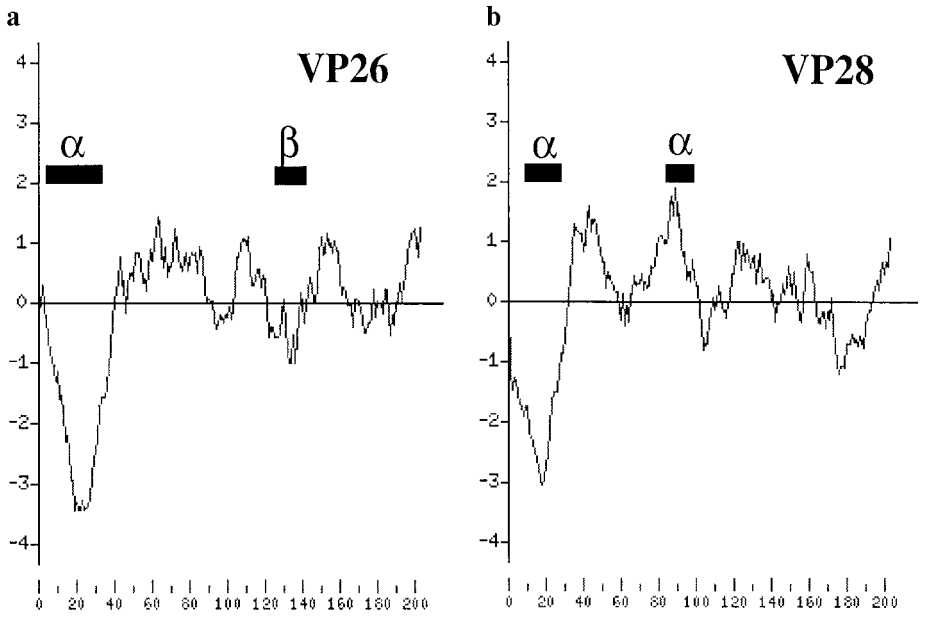

FIG. 3. Hydrophobicity plots of VP26 (a) and VP28 (b). The amino acid number is on the abcis; the hydrophobicity value on the ordinate. α helices (■) and β sheets (■) are indicated.

used to generate recombinant baculoviruses expressing the putative WSSV virion proteins in insect cells. The VP26 and VP28 genes were cloned downstream of the polyhedrin promoter in the plasmid pFastBac-D/GFP, which contains a GFP gene downstream of the p10 promoter. The recombinant viruses generated from pFastBac-D/GFP (control), and the plasmids with VP26 and VP28 were designated *Ac*MNPV-GFP, *Ac*MNPV-WSSVvp26, and *Ac*MNPV-WSSVvp28, respectively. All recombinant viruses expressed GFP off the p10 promoter to facilitate detection and titration; the latter two also expressed VP26 and VP28, respectively, off the polyhedrin promoter.

Extracts of *Sf*21 cells infected with *Ac*MNPV-wt, *Ac*MNPV-GFP, *Ac*MNPV-WSSVvp26, and *Ac*MNPV-WSSVvp28 were analyzed on a 15% SDS–PAGE gel. In cells infected with wild-type *Ac*MNPV (Fig. 4a, lane 3), a 32-kDa band that represented polyhedrin was visible. In the lanes containing extracts of *Ac*MNPV-GFP infected cells (lane 4) and cells infected with the recombinants expressing WSSV proteins (lanes 5 and 6), a GFP protein band was observed at ~29 kDa. The GFP expression in the cells infected with *Ac*MNPV-GFP was stronger than the GFP expression in recombinant virus producing WSSV proteins from the polyhedrin promoter (lanes 5 and 6). This was also readily observed after UV illumination of cells infected with the various *Ac*MNPV recombinants, where the fluorescence of GFP in *Ac*MNPV-GFP-infected cells was the strongest (not shown). The expression of the WSSV proteins from the polyhedrin promoter was significantly higher than the expression of GFP from the p10 promoter (lane 5 and 6). Strong expression of a 22-kDa protein was observed in extracts of *Ac*MNPV-WSSVvp26-infected cells, most likely representing WSSV VP26 (lane 5). Cells infected with *Ac*MNPV-WSSVvp28 (lane 6) showed a strong expression of a 28-kDa protein. The position of GFP in these gels was confirmed by Western analysis using anti-GFP antiserum (data not shown).

Western analysis was performed on samples from *Sf*21 cells infected with wild-type and recombinant *Ac*MNPV. A polyclonal antibody against WSSV virions was used to detect recombinant VP26 and VP28 (Fig. 4b). Both VP26 and VP28 were detected in these cell extracts. VP26 was detected at 22 kDa, in conformity with the Coomassie-Brilliant-Blue-stained gel (Figs. 4a, lane 5, and 4b, lane 5). This is identical to its theoretical size but lower compared to the position of VP26 when directly extracted from WSSV virions. VP28 migrated at the same position as VP28 from WSSV virions, which is significant higher than the theoretical size of 22 kDa for this protein. The polyclonal antibody did not show major cross reactivity with insect cells (lane 2) or baculovirus proteins (lanes 3 and 4), as observed from the very low background reaction in these samples.

Relatedness of VP26 and VP28

Homology searches with WSSV VP26 and VP28 were performed against GenBank/EMBL, SWISSPROT, and PIR databases using FASTA, TFASTA, and BLAST. No significant homology with baculovirus envelope or capsid proteins or with structural proteins from other large

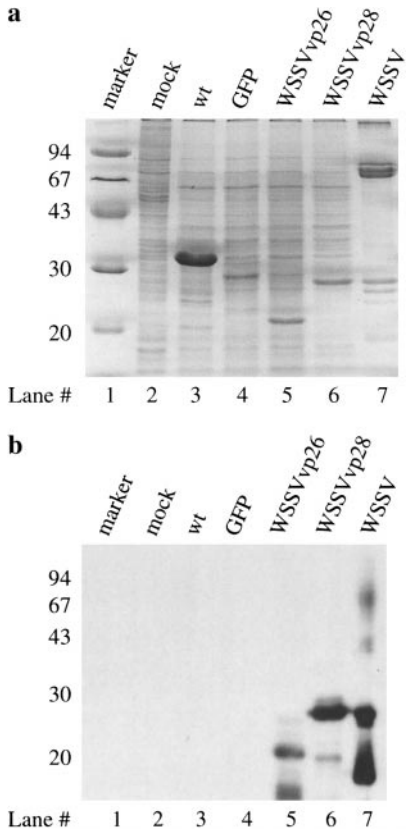

FIG. 4. Baculovirus expression of WSSV structural proteins in insect cells. (a) Coomassie-Brilliant-Blue-stained 15% SDS–PAGE gel with low molecular weight protein markers (lane 1), with extracts of *Sf*21 cells (lane 2), infected with wild-type *Ac*MNPV infection (lane 3), with *Ac*MNPV-GFP (lane 4), with *Ac*MNPV-WSSVvp26 (lane 5) and *Ac*MNPV-WSSVvp28 (lane 6), and with purified WSSV-virions (lane 7). (b) Western blot of the SDS–PAGE of a. WSSV polyclonal antiserum is used and detection is performed with the ECL kit.

DNA viruses could be found with the sequences in the GenBank.

DISCUSSION

Four major and several less prominent protein bands were observed, when purified WSSV virions were analyzed in SDS–PAGE (Fig. 1). The major protein bands had approximate sizes of 19 kDa (VP19), 24 kDa (VP24), 26 kDa (VP26), and 28 kDa (VP28), respectively. Only two of these, VP24 and VP26, were observed in the nucleocapsid preparation, suggesting that these are the major components of the nucleocapsid of WSSV. The other major virion proteins, VP19 and VP28, most likely are constituents of the virion envelope or the tegument, as they were stripped off the virion by NP40 treatment. The sizes found for the major virion proteins were similar to those described by Hameed *et al.* (1998) and Nadala *et al.* (1998). A major difference is the number of protein bands observed. Hameed *et al.* (1998) and Nadala *et al.* (1998) observed three major proteins in the range of 18–28 kDa, whereas we observed four major proteins in this size range (Fig. 1). Whether this difference is due to variation in WSSV isolates or the result of the WSSV purification procedure remains to be investigated. The 19-kDa and a 27.5-kDa protein that Nadala *et al.* (1998) observed in the envelope fraction have the same size as VP19 and VP28 from our envelope fraction and may therefore be the same proteins. Furthermore these authors observed a 23.5-kDa protein in the nucleocapsid fraction that may correspond to VP24 or VP26, both of which are found in the nucleocapsid fraction.

Two of the major structural WSSV proteins, one from the envelope fraction (VP28) and one from the nucleocapsid preparation (VP26), were selected for further analysis to study, in particular, their relatedness to structural proteins of other viruses including baculoviruses. The N-terminal amino acid sequence of these proteins was used to locate the ORFs coding for these proteins on the WSSV genome by direct sequencing (VP26) or by using degenerated primers and colony lifting (VP28). As such these are the first WSSV virion proteins whose genes have been identified. Antibodies against VP26 and/or VP28 are being generated and may serve as a specific diagnostic reagent to detect WSSV infection in shrimp. Furthermore these antibodies could be used in immunogold labeling of virus particles. Such a study would provide visualized evidence that the proteins are indeed structural virion proteins and exclude the possibility that they are fortuitously associated with the virus particles.

The ORFs of *vp26* and *vp28* coded for proteins with theoretical sizes of 22 kDa. The authentic N-terminal amino acid sequences of VP26 and VP28 were confirmed by direct protein sequencing. Both protein sequences contain multiple putative *N*- and *O*-glycosylation sites as well as phosphorylation sites (Figs. 2a and 2b). These theoretical sizes differed by ~4 and 6 kDa, respectively, from their mobility in Coomassie-Brilliant-Blue-stained SDS–PAGE gels and can be explained either by post-translational modifications such as glycosylation and phosphorylation or by splicing. Expression in the baculovirus insect cell system was performed to determine whether the ORFs encoded the major WSSV structural virion proteins and to confirm the identity and coding capacity of these virion proteins. Furthermore the immunoreactivity of these proteins with a WSSV- and *Ac*MNPV-specific polyclonal antisera was investigated. Both VP26 and VP28 were highly expressed from the polyhedrin promoter in cells infected with recombinant *Ac*MNPV (Fig. 4). The theoretical size of the protein encoded by the *vp28* ORF was 22 kDa. Expression in insect cells resulted in a protein band of 28 kDa, which is the same size as the protein from WSSV isolated from infected shrimp. This suggests that posttranslational

modifications of this protein may have been correctly performed in insect cells.

When VP26 was expressed, a band of 22 kDa was observed. This is similar to the VP26 theoretical size but 4 kDa smaller than its authentic counterpart protein from purified WSSV (Fig 4). This suggests that the *vp26* gene product is made but not further processed in insects cells. Additional experiments are needed to show which posttranslational modifications have occurred on these WSSV structural proteins in WSSV virions, and how processing is performed in the insect cells. Another possibility is that WSSV VP26 is derived from a spliced mRNA specifying a larger peptide. In that case the genomic *vp*26 sequence presented here only partially codes for the correct protein. This could mean that the 3′ end of the expressed protein does not correspond to the 3′ end of the authentic VP26. In the *vp*26 sequence, several splicing donor sites are present, e.g., at position 177 (AGGTAATG). To determine whether VP26 is the result of splicing, the VP26 sequence could be determined from the mRNA using cDNA technology such as 3′ RACE. The recombinant WSSV VP26 and VP28 can be used to generate monospecific polyclonal antibodies for studies on the structure and morphogenesis of WSSV virions.

Linear and circular membranous structures appear to be formed *de novo* in the nucleoplasm of cells of WSSV-infected shrimps (Durand *et al.,* 1997). Similar virus induced intranuclear microvesicles are also found in the nucleus of baculovirus infected cells where *de novo* synthesis of viral envelopes occurs (Williams and Faulkner, 1997). The viral envelope protein encoded by *Ac*MNPV-*orf46* contains a hydrophobic domain on the extreme N terminus that is sufficient to direct this protein into these intranuclear microvesicles (Hong *et al.,* 1997). Both VP26 and VP28 of WSSV virions have a hydrophobic domain on their N terminus, which might have a similar signal function for transport and also may be responsible for the membranous structures associated with WSSV infection *in vivo* (Durand *et al.,* 1997). VP26, the major protein of the nucleocapsid, appears to be a very basic protein with a theoretical isoelectric point of 9.4. Because of its basic character, it may have a close association with the viral DNA.

The objective of this study was also to reveal possible homology of WSSV virion proteins with structural proteins of other viruses including baculovirus. Western analysis confirmed the WSSV origin of the expressed VP26 and VP28. Both peptides gave a strong reaction with the polyclonal antiserum against purified WSSV (Fig. 4b). The absence of any reaction of this WSSV antiserum with proteins of wild-type *Ac*MNPV infected cells also indicated that there is no serological relationship between WSSV virion proteins and baculovirus proteins. Baculovirus structural protein do share antigenic determinants (Smith and Summers, 1981). When the WSSV nucleocapsid protein (VP26) and the envelope or tegument protein (VP28) sequences are compared with proteins known to be present in the baculovirus virion (e.g., *Ac*MNPV-gp41, *Ac*MNPV-vp39) or envelope (e.g., *Ac*MNPV-gp64, *Ac*MNPV-p25) and other sequences available in the GenBank, no significant homologies were found. From this analysis we conclude that the WSSV virion structural proteins described in this paper do not have any homology with baculovirus structural proteins. However, it cannot be excluded that other WSSV virion proteins, such as VP24 and VP19, may show homology to baculovirus proteins.

In the latest taxonomic revision of viruses by the International Committee on Taxonomy of Viruses (Murphy *et al.,* 1995), WSSV has not been classified due to the lack of molecular information. In a previous report (Francki *et al.,* 1991), WSSV was classified as a nonoccluded baculovirus based on the rod to bacilliform shape of the WSSV virion. Ongoing sequence analysis (35,000 bp) of the WSSV genome so far indicated that WSSV has only very limited (*rr* genes), if any, homology with baculovirus sequences to date, or with any other known virus or organism. In contrast, baculoviruses do exhibit homology of a high proportion (50%) of their genes, including those encoding virion structural proteins (Hu *et al.,* 1998; Kuzio *et al.,* 1999). The two major WSSV structural proteins investigated here do not share any homology with baculovirus virion proteins. This supports the view that WSSV may be a representative of a new baculovirus genus (whispovirus) or a new virus family (proposed name Whispoviridae). The recent results on the phylogenetic analysis of *rr* genes of WSSV (van Hulten *et al.,* 2000) give further credence to the latter view.

MATERIALS AND METHODS

White Spot Syndrome Virus production and purification

The virus used in this study was isolated from infected *P. monodon* shrimp from Thailand. Infected tissue was homogenized in TN buffer (20 mM Tris–HCl and 400 mM NaCl, pH 7.4). After centrifugation at 1700 *g* for 10 min, the supernatant was filtered (0.45-μm filter) and injected intramuscularly into healthy *P. monodon* in the lateral area of the fourth abdominal segment to initiate infection. After 4 days haemolymph was withdrawn from moribund shrimps and mixed with modified Alsever solution (Rodriguez *et al.,* 1995) as an anticoagulant. After dilution in TNE (20 mM Tris–HCl, 400 mM NaCl, and 5 mM EDTA, pH 7.4) the haemolymph was clarified from haemocytes at 1700 *g* for 10 min at 4°C. The virus particles were then sedimented by centrifugation at 45,000 *g* at 4°C for 1 h and resuspended in TN buffer.

The virus envelope was removed from the virus particles by treatment with Nonidet-P40 (N-P40). One percent

N-P40 was added to the virus solution and incubated for 30 min at room temperature with gentle rocking. The nucleocapsids were sedimented at 80,000 *g* for 30 min at 4°C. The pellet was dissolved in TE (10 mM Tris–HCl and 1 mM EDTA, pH 7.5). The proteins in the supernatant were acetone-precipitated and resuspended in TE.

Electron microscopy

For transmission electron microscopy (TEM), the virus suspension was mounted on Formvar-coated, carbon-stabilized nickel grids (400 mesh) and negatively stained with phosphotungstic acid (2% PTA). The specimens were examined in a Philips CM12 electron microscope.

Nucleic acid purification

Viral DNA was isolated from purified virions by treatment with proteinase K (0.2 mg/ml) and sarcosyl (1%) at 45°C for 3 h, followed by phenol/chloroform extraction and dialysis against TE (10 mM Tris–HCl and 1 mM EDTA, pH 7.5). The purity and concentration of the DNA was determined by agarose gel electrophoresis.

Plasmid constructions

WSSV subgenomic fragments were generated by restriction enzyme analysis, cloned into pBluescript SK+ (Stratagene) and transformed into *Escherichia coli* DH5α using standard techniques (Sambrook *et al.,* 1989). DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, and colony lifting were carried out according to standard protocols (Sambrook *et al.,* 1989). PCR was performed using custom designed and synthesized primers. DNA encoding the N terminus of *vp28* was amplified by PCR from total WSSV DNA using degenerated primers based on the N-terminal amino acid sequence of VP28. The forward primer used was 5′ CA*GAATTC*TCDATNGTYTTNGTNAC 3′ and the reverse primer was 5′ CA*GAATTC*ATGGAYYTNWSNTTYAC 3′ with *Eco*RI sites (italics) (D = A, T, or G; N = A, C, G, or T; Y = C or T; W = A or T; S = C or G).

DNA sequencing and computer analysis

Plasmid DNA for sequencing was purified using the QIAprep Miniprep System or JETstar Plasmid Purification System (Qiagen, Inc.). Sequencing was performed using the universal pBluescript forward and reverse primers and custom primers for both strands. Automatic sequencing was carried out using an Applied Biosystems automated DNA sequencer (Eurogentec, Belgium).

The generated sequences were analyzed with UWGCG computer programs (release 10.0). The DNA and the deduced amino acids sequences were compared with GenBank/EMBL, SWISSPORT, and PIR databases using the programs FASTA, TFASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.,* 1997).

Cells and viruses

Spodoptera frugiperda (*Sf*-AE-21) cells (Vaughn *et al.,* 1977) were cultured in Grace's insect medium (GIBCO BRL) supplemented with 10% foetal calf serum (FCS). The E2 strain of *Autographa californica* multicapsid nucleopolyhedrovirus (*Ac*MNPV) (Summers and Smith, 1978) was used as wild-type (wt) virus. Routine cell culture maintenance and virus infection procedures were carried out according to published procedures (Smith and Summers, 1987; King and Possee, 1992).

Engineering of recombinants

The Bac-to-Bac system (GIBCO BRL) was employed to overexpress WSSV VP26 and VP28 in insect cells. To facilitate detection and titration of Bac-to-Bac recombinants upon infection of insect cells, the Green Fluorescent Protein (GFP) gene was introduced into the pFastBac-DUAL vector downstream of the p10 promoter. The GFP gene was removed from plasmid pVL92GFP (Reilander *et al.,* 1996) after digestion of this plasmid with *Xba*I and *Kpn*I. The 700-bp GFP-containing fragment was isolated by agarose gel electrophoresis and GlassMAX purification (GIBCO BRL), blunt-ended using DNA polymerase, and inserted into the *Sma*I site of multiple cloning region II of pFastBac-DUAL downstream of the p10 promoter. The resulting plasmid was named pFastBac-D/GFP and contained region I for insertion of a foreign gene downstream of the polyhedrin promoter. Recombinant virus, expressing only the GFP from the p10 promoter, was constructed according to the Bac-to-Bac system protocol (GIBCO BRL) and the resulting virus was designated *Ac*MNPV-GFP.

PCR was performed on the WSSV plasmids containing the putative complete open reading frames (ORFs) of *vp26* and *vp28*. A *Bam*HI site was introduced at the 3′ end of the ORFs and a *Hin*dIII site at the 5′ end. *Vp26* and *vp28* were first cloned into the pET28a vector (Novagen), excised with *Bam*HI and *Not*I, and inserted downstream of the polyhedrin promoter of plasmid pFastBac-D/GFP. The resulting plasmids were named pFastBac-D/G-vp26 and pFastBac-D/G-vp28, respectively. Recombinant viruses expressing the GFP from the p10 promoter and VP26 or VP28 from the polyhedrin promoter were constructed according to the Bac-to-Bac system protocol (GIBCO BRL), and the viruses were designated *Ac*MNPV-WSSVvp26 and *Ac*MNPV-WSSVvp28, respectively.

SDS–PAGE, protein sequencing, and immunoblotting

Insect cells infected with wild-type *Ac*MNPV, insect cells infected with recombinant *Ac*MNPV expressing heterologous proteins (GFP, VP26, VP28), and purified WSSV

were analyzed in 15% SDS–PAGE gels as described in Laemmli (1970). Proteins were visualized using Coomassie Brilliant Blue staining. Semi-dry blotting was performed onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using a CAPS buffer (10 mM CAPS in 10% methanol), or onto an Immobilon-P (Millipore) using a Tris–glycine buffer [25 mM Tris base and 192 mM glycine, 10% (v/v) methanol, pH 8.3]. Proteins were visualized on the PVDF membrane using Coomassie Brilliant Blue staining. Major protein bands from WSSV virion preparations were excised from the filter and N-terminal sequenced (ProSeq. Inc., MA).

Immobilon-P membranes were blocked in 2% low-fat milk powder (Campina, the Netherlands) in TBS (0.2 M NaCl and 50 mM Tris–HCl, pH 7.4). Immunodetection was performed by incubation of the blot in a polyclonal rabbit anti-WSSV serum (a gift from Prof. P. C. Loh, University of Honolulu, HI) diluted 1:2000 in TBS with 0.2% low-fat milk powder for 1 h at room temperature. Subsequently, anti-rabbit antibody conjugated with horseradish peroxidase (Amersham) was used at a concentration of 1:2000 and detection was performed with an enhanced chemiluminescent-light detection kit (Amersham).

ACKNOWLEDGMENTS

The polyclonal rabbit anti-WSSV serum was a generous gift from Dr. Ph. Loh, University of Honolulu, Hawaii. This paper is dedicated to Dr. Jan H. Boon († 1997), Laboratory of Fisheries, Agricultural University, who initiated this study. This research was supported by Intervet International BV, Boxmeer, The Netherlands, through financial aid from the Ministry of Economic Affairs.

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